

Involvement of Cdk5/p25 in Digoxin-triggered Prostate Cancer Cell Apoptosis*

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Cardiac digitalis has been considered to be a treatment for breast cancer. Our previous study indicates that digoxin, one member in digitalis, decreases the proliferation of prostate cancer cells, but the mechanisms remain unclear. In the present study, Ca^{2+} proved to be an important factor in digoxin-triggered prostate cancer cell death. Because cyclin-dependent kinase (Cdk5) and p35 cleavage (p25 formation) have been reported to be targets of intracellular Ca^{2+} , and subsequently correlated to apoptosis, we not only demonstrated first that Cdk5, p35, and p25 proteins were all expressed in prostate cancer cells (including lymph node carcinoma of the prostate (LNCaP) and DU-145 cells), but also showed where p25 formation and Cdk5 kinase activity were affected by treatment with digoxin. The inhibitor of p35 cleavage (calpeptin) was used to reduce p25 formation, and the result suggested that p25 accumulation might be the major cause of digoxin-triggered LNCaP cell death. Butyrolactone-I and roscovitine, two Cdk5 kinase inhibitors, were also found to prevent digoxin-triggered LNCaP cell death. In addition, treatment of siRNA-Cdk5 diminished digoxin-triggered cell death, as compared with the treatments of siRNA-Cdk1 or siRNA-Cdk2, which implies the specific involvement of Cdk5 in digoxin-triggered cell death. Caspase inhibitor set and terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling assay were used to demonstrate that digoxin-triggered LNCaP cell apoptosis through Cdk5 activation. These results suggest that Cdk5/p35 and p25 are novel players in digoxin-triggered prostate cancer cell apoptosis and, therefore, become potential therapeutic targets.

Prostate cancer is a common malignancy and age-related cancer in men (1). Although the cancer marker prostate-specific antigen helps to identify prostate cancer, the lethality is still very high. Traditionally, before prostate cancer becomes androgen-independent, the therapy is gonadectomy. Once prostate

cancer has become androgen-independent, definitive radio- or chemotherapies are used.

Digoxin, a purified digitalis preparation derived from the leaves of *Digitalis lanata* (foxglove), plays a vital role in the therapy of congestive heart failure (2) and is the most widely used digitalis glycoside. It has direct and indirect cardiovascular actions for treating heart failure (3). Epidemiological studies have shown the anti-cancer effects of digitalis (4). Breast cancer patients treated with digitalis have been found to have a lower death rate than non-treated patients (4). Five years after mastectomy, cancer recurrence rates in control patients who were not given digitalis increased by 9.6-fold, as compared with patients who were treated with digitalis (5). Our previous study demonstrated that digoxin is able to induce the rise of intracellular Ca^{2+} and the toxicity of prostate cancer cells, but detailed mechanisms still remain unknown (6).

Cdk5 is a unique member of a small serine/threonine cyclin-dependent kinase (cdk)¹ family. Although most Cdks are involved in cell-cycle regulation, Cdk5 has connections in neuronal apoptosis and degeneration (7). The physiological roles for Cdk5 are regulating neuronal cytoskeleton, axon guidance, membrane transport, synaptic function, dopamine signaling, and drug addiction. Like other Cdks, Cdk5 shows no kinase activity and requires association with a regulatory partner, such as p35, for activation and maintains a normal physiology of neurons (8). In the Alzheimer's model, Cdk5 seems to be activated and leads to neuronal death under oxidative stress from various sources, such as amyloid β peptides and the accumulation of intracellular Ca^{2+} (9). The p25, a Ca^{2+} -dependent p35 cleavage product, is believed to be a highly regulating Cdk5 kinase activity and plays an important role in Alzheimer's pathology (10). Recently, Cdk5/p35 was reported to distribute in cells of several reproductive glands, such as Leydig cells, Sertoli cells, and the prostate gland, and wherever Cdk5 kinase is active (11–14). In those cells, Cdk5 kinase activity is regulated by luteinizing hormone and follicle-stimulating hormone, the trophic hormones for the secretion of androgen (12), and contributes to the growth of the prostate gland. Moreover, it was reported recently (15) that Cdk5 kinase activity is affected by flavonoids, prominent plant secondary metabolites, which reveals the connections between Cdk5 and plant extracts. Therefore, it is interesting to investigate whether Cdk5 is involved in digoxin-caused prostate cancer cell toxicity. Our results indicate that digoxin-triggered prostate

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¹ The abbreviations used are: cdk, cyclin-dependent kinase; siRNA, short interfering RNA; LNCaP, lymph node carcinoma of the prostate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide; PBS, phosphate-buffered saline; BL-I, butyrolactone-I; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; VAD-fmk, VAD-fluoromethyl ketone.

cancer cell apoptosis resulted primarily from p25-dependent activation of Cdk5.

MATERIALS AND METHODS

Cell Culture and Transfection of siRNA—Prostate cancer cell lines, lymph node carcinoma of the prostate (LNCaP) and DU-145 (BCRC 60088 and BCRC 60348) were provided from Culture Collection and Research Center, Food Industry Research and Development Institute, Taiwan, Republic of China. LNCaP cells were cultured in RPMI 1640 medium (Sigma) plus 10% fetal bovine serum (Hyclone), and penicillin/streptomycin (Life Technologies, Inc.-Life Technologies, Inc.) at 37 °C in a humidified atmosphere at 5% CO₂. DU-145 cells were cultured in MEM medium (Sigma) plus 10% fetal bovine serum (Hyclone), 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, and penicillin/streptomycin (Biochrom AG) at 37 °C in a humidified atmosphere at 5% CO₂. siRNA-Cdk5, siRNA-Cdk1, siRNA-Cdk2, and nonspecific control of siRNA were purchased from Upstate Biotechnology (Dharmacon) which are SMARTpool™ containing four pooled SMARTselected siRNA duplexes. Introduction of siRNAs into LNCaP cells was performed by using LipofectAMINE 2000 (Invitrogen) with 50 pmol siRNA/10⁵ cells 2 days before treatment with digoxin.

Cell Viability Assay—The modified colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was manipulated to quantify the viability of LNCaP cancer cells (16). Yellow MTT compound (Sigma) was converted by living cells to form blue formazan, which is soluble in dimethyl sulfoxide (Me₂SO). The intensity of blue staining in culture medium proportionally represented the number of living cells and was measured by optical density reader (SpectroMAX plus, Molecular Devices) at 570 nm (background = 630 nm).

Immunoprecipitation and Western Blot Analysis—Cell lysate was produced in lysis buffer (20 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 137 mM NaCl, 50 μM EDTA, protease inhibitor mixture, and 1 mM phenylmethylsulfonyl fluoride) or extract buffer (100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM Na₄P₂O₇, 2 mM Na₃VO₄, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor mixture) for Cdk5 immunoprecipitation. Cell extracts were immunoprecipitated and immunoblotted. Immunoprecipitates were collected by binding to protein G PLUS/protein A-agarose (Oncogene Research Products). Proteins were analyzed by direct Western blotting (30 μg/lane) or blotting after immunoprecipitation (1–2 mg per immunoprecipitation). Antibodies used included anti-Cdk5 antibody, anti-Cdk1 antibody, anti-Cdk2 (all Upstate Biotechnology), anti-p35 antibody (Santa Cruz Biotechnology), anti-actin (MAB1501, Chemicon), and peroxidase-conjugated anti-mouse or anti-rabbit antibodies (Jackson ImmunoResearch Laboratory). ECL detection reagent (NEN) was used to detect the immunoreactive proteins.

Cdk5 Kinase Assay—Kinase assay was performed by washing immunoprecipitates three times with kinase reaction buffer (50 mM HEPES, pH 7.0, 10 mM MgCl₂, and 1 mM dithiothreitol). The protein G PLUS/protein A-agarose beads with target proteins were incubated with kinase reaction buffer containing 2 μg of substrate (histone H1, Upstate Biotechnology) and 10 μCi of [³²P]ATP in a final volume of 40 μl at 30 °C for 30 min.

Staining—LNCaP cells that were cultured on coverslips were fixed for 10 min in 4% paraformaldehyde and 2% sucrose in phosphate-buffered saline (PBS) at room temperature after washing with PBS twice. Fixed cells were then washed once again with PBS. The buffer containing 3% bovine serum albumin and 0.3% Triton X-100 in PBS was then added and mixed for 2 min at room temperature. After discarding the buffer, PBS-washing and subsequent 3% bovine serum albumin-PBS blocking were done for another 15 min at room temperature. The terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) staining was carried out using the *in situ* Cell Death Detection Kit (Roche Applied Science). The slides were incubated in the TUNEL reaction mixture (including FITC-conjugated modified nucleotides and terminal deoxynucleotidyl transferase) for 1 h at 37 °C. Phase images and TUNEL signals of cells were visualized directly under Nikon microscopy with an fluorescein isothiocyanate filter.

Statistics—All values are given as the means ± S.E. In some cases, means were tested for homogeneity by two-way analysis of variance, and the difference between specific means were tested for significance by Duncan's multiple-range test (17). In all other cases, a Student's *t* test was used. A difference between two means was considered statistically significant when *p* < 0.05.

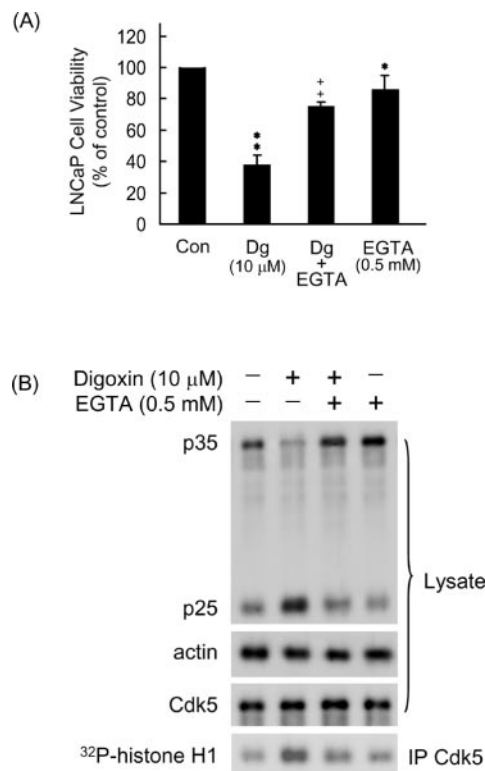


FIG. 1. Digoxin triggers Ca²⁺-dependent LNCaP cell death and p25-dependent Cdk5 activation. LNCaP cells were treated as follows: control, digoxin (Dg, 10 μM), digoxin + EGTA, and EGTA (0.5 mM) for 2 days. *A*, cell viability was measured by MTT assay as described under "Materials and Methods" (*n* = 4). Control value = 100%; *, *p* < 0.05 and **, *p* < 0.01, versus control group; ++, *p* < 0.01 versus digoxin group. *B*, in p35 cleavage study, cell lysates were directly immunoblotted by anti-p35 antibody (C-terminal) and anti-actin. In the Cdk5 kinase assay, cell lysates were subjected to Cdk5 immunoprecipitation followed by anti-Cdk5 immunoblotting and *in vitro* [³²P]ATP kinase assay using histone H1 as the substrate.

RESULTS

Digoxin Triggers Ca²⁺-dependent Prostate Cancer Cell Death and p25-dependent Cdk5 Activation—Our previous study reports that digoxin-induced toxicity of prostate cancer cells is accompanied by the increase of intracellular Ca²⁺ (6), which is believed to be a key factor in apoptosis (18). Thus, we chelated Ca²⁺ from culture medium by EGTA (0.5 mM for 2 days) and found that digoxin-triggered (10 μM for 2 days) cell death was significantly prevented (Figs. 1*A* and 2*A*), although there were slight decreases of cell numbers in EGTA-only groups. Cell viability was measured by MTT assay, as described under "Materials and Methods" (16). The prostate cancer cell lines tested included LNCaP cells and DU-145 cells (see Figs. 1 and 2, respectively). In the Alzheimer's model, Cdk5 is activated by various signals such as β-amyloid peptide and leads to neuronal death; much evidence indicates that Ca²⁺ accumulation and subsequent p35 cleavage are correlated to Cdk5 activation (10). In addition to the presence of Cdk5/p35 complex in reproductive tissues (11–14), it would be interesting to explore the protein expressions of Cdk5/p35 and p25 in prostate cancer cells. Immunoblotting assay was used to detect protein expressions such as anti-Cdk5 (Upstate Biotechnology) and anti-p35/p25 (C-terminal, sc-820, Santa Cruz Biotechnology). Direct *in vitro* Cdk5 kinase assay using histone-H1 as substrate after immunoprecipitation of Cdk5 was also performed. The results showed that p25 formation and Cdk5 kinase activity were both stimulated by digoxin (Figs. 1*B* and 2*B*, lane 2), whereas actin and Cdk5 protein expressions were not affected. Treatment of EGTA diminished digoxin-induced p25 formation and Cdk5

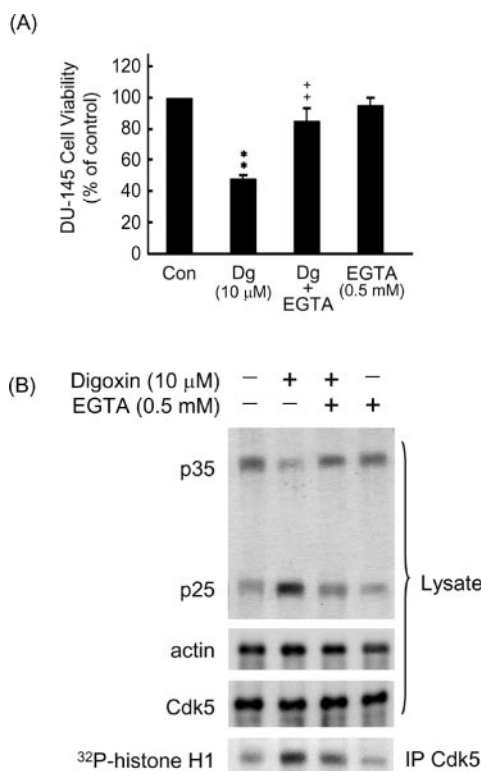


FIG. 2. Digoxin triggers Ca^{2+} -dependent DU-145 cell death and p25-dependent Cdk5 activation. DU-145 cells were treated as follows: control, digoxin (*Dg*, 10 μM), digoxin + EGTA, and EGTA (0.5 mM) for 2 days. **A**, cell viability was measured by MTT assay as described under "Materials and Methods" ($n = 4$). Control value = 100%; **, $p < 0.01$ versus control group; ++, $p < 0.01$ versus digoxin group. **B**, in p35 cleavage study, cell lysates were directly immunoblotted by anti-p35 antibody (C-terminal) and anti-actin. In Cdk5 kinase assay, cell lysates were subjected to Cdk5 immunoprecipitation followed by anti-Cdk5 immunoblotting and *in vitro* [^{32}P]ATP kinase assay using histone H1 as the substrate.

activation. This result suggests that digoxin stimulates Cdk5 activity through Ca^{2+} -dependent cleavage of p35 into p25 (Figs. 1B and 2B, lane 3).

p25 Formation Is Vital in Digoxin-triggered LNCaP Cell Death—In previous results, we found that digoxin might activate Cdk5 through p35 cleavage in prostate cancer cells. Thus, it was interesting to examine whether inhibition of p25 formation could affect digoxin-triggered cell death. Inhibitor of p35 cleavage, calpeptin (Calbiochem), was used in the study by Lee *et al.* (21) and was found able to significantly prevent digoxin-triggered LNCaP cell death in a dose-dependent manner (Fig. 3A). Treatment of digoxin with calpeptin (15 μM) caused a 12% drop in cell number versus control group, as compared with a 62% drop by the digoxin group (Fig. 3A). In Fig. 3B, Cdk5 kinase activity certainly dropped by the inhibition of p35 cleavage under treatment of digoxin, whereas the protein expressions of actin and Cdk5 were not affected. These results suggest that p25 formation plays an important role in digoxin-triggered LNCaP cell death.

Cdk5 Kinase Activity Correlates to Digoxin-triggered LNCaP Cell Death—To understand the effects of Cdk5 kinase activity directly upon digoxin-triggered cell death, two commonly used Cdk5 kinase inhibitors (butyrolactone-1 (BL-I) and roscovitine (RV), Calbiochem) were co-treated with digoxin, and cell viability was monitored. BL-I and RV are both relatively specific for inhibiting Cdk5 kinase activity and are frequently applied in Cdk5-related studies (19, 20, 22). The data indicated that both inhibitors prevented digoxin-triggered cell death in a dose-dependent manner, but the two results were slightly different

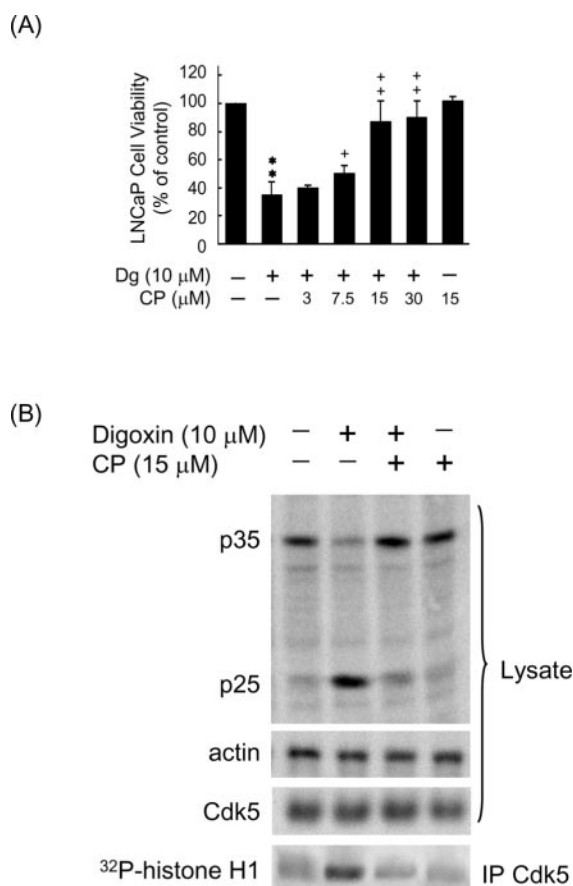


FIG. 3. p35 cleavage is important to digoxin-triggered LNCaP cell death. **A**, different dosages of p35 cleavage inhibitor (calpeptin, 0 ~ 30 μM) were added in LNCaP cell culture medium with or without digoxin (10 μM) for 2 days. Cell viability was measured by MTT assay as described under "Materials and Methods" ($n = 4$). Control value = 100%; **, $p < 0.01$ versus control group; +, $p < 0.05$ and ++, $p < 0.01$, versus digoxin group. **B**, LNCaP cells were treated with 15 μM calpeptin for 2 days to investigate the effects of p25 formation on digoxin-triggered Cdk5 activation. Cell lysates were directly immunoblotted by anti-p35 antibody (C-terminal) and anti-actin. In Cdk5 kinase assay, cell lysates were subjected to Cdk5 immunoprecipitation followed by anti-Cdk5 immunoblotting and *in vitro* [^{32}P]ATP kinase assay using histone H1 as the substrate.

from each other (Fig. 4, left panels). Two treatments with 10 μM inhibitor were used as the efficient dosage to inhibit Cdk5 kinase activity, which was confirmed by *in vitro* kinase assay (Fig. 4, right panels). Consequently, butyrolactone-1 was suggested as a better inhibitor for further investigation of Cdk5-related experiments.

Digoxin-triggered Cell Death Is Cdk5 Protein-dependent—On the basis of digoxin-triggered cell death caused by Cdk5 kinase activity, elimination of Cdk5 protein has become another quick way to demonstrate how Cdk5 gets involved in digoxin-triggered cell death. Cdk5 protein expression was knocked-down by siRNA (Fig. 5A, white bars), whereas siRNA-*Cdk1* (Fig. 5A, hatched bars) and siRNA-*Cdk2* (Fig. 5A, gray bars) were also performed. The data indicated that reduction of Cdk5 protein expression mitigated LNCaP cell death caused by digoxin (Fig. 5A, compare bars 4 and 2), although cell viability was affected by treatment of siRNA-*Cdk5* alone (Fig. 5A, compare bars 3 and 1). The black bars in Fig. 5A were control groups of siRNA treatment, which was transfected with nonspecific siRNA (Upstate Biotechnology). There was no significant difference in cell viability between the nonspecific siRNA group and the non-transfected group (data not shown). The groups treated with siRNA-*Cdk1* and siRNA-*Cdk2* showed no significant change in

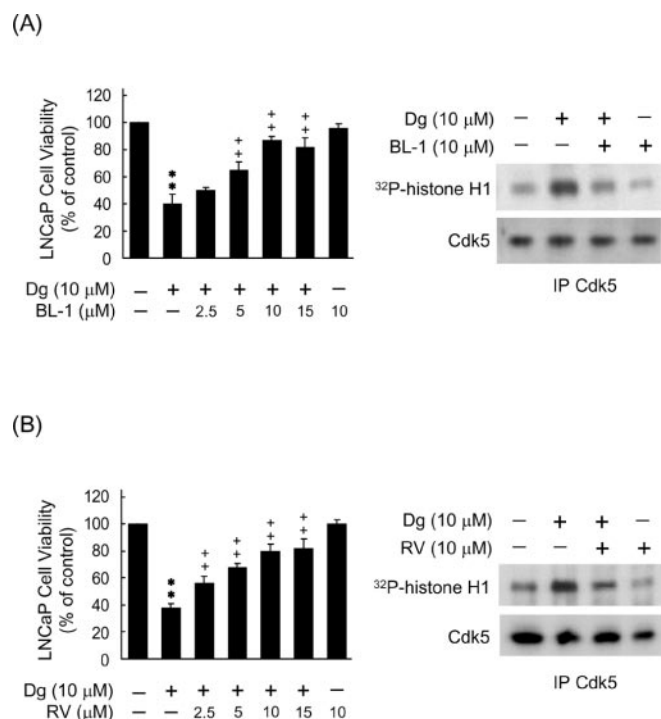


FIG. 4. Cdk5 kinase activity correlates to digoxin-triggered LNCaP cell death. Dose-dependent effects of BL-I (A) and RV (B) on digoxin-affected LNCaP cell viability and Cdk5 activation were monitored after 2-day treatment. Cell viability was measured by MTT assay as described under "Materials and Methods" ($n = 4$). Control value = 100%; **, $p < 0.01$ versus control group; ++, $p < 0.01$ versus digoxin group. Cell lysates were subjected to Cdk5 immunoprecipitation followed by anti-Cdk5 immunoblotting and *in vitro* [32 P]ATP kinase assay using histone H1 as the substrate.

cell death with or without the treatment of digoxin (Fig. 5A, bars 5-8). Fig. 5B indicates the efficacies of the three treatments of siRNA by immunoblotting LNCaP cell lysates in which Cdk5 kinase assay was also performed. Taken together, these results illustrate that Cdk5 protein plays an important role in digoxin-triggered LNCaP cell death.

Digoxin-triggered LNCaP Cell Death Is Cdk5-related Apoptosis—Based upon our previous results, digoxin triggers Cdk5 protein/activity-dependent cell death. In accordance with the direct link between Cdk5 and apoptosis (23, 24), we continued to verify whether digoxin could cause LNCaP cell apoptosis by affecting Cdk5. Caspase-family inhibitor set (1:1000 dilution, Chemicon) was used to test the cytotoxicity of digoxin by direct addition to culture medium. The effects of synthetic peptide inhibitors of caspases which irreversibly inhibit the activity of each caspase-family proteases are indicated in Fig. 6A. The data reveal that at least caspase 8, 3, and 6 were involved in digoxin-triggered LNCaP cell death (Fig. 6B). In addition, staining by TUNEL (Roche Applied Science) was used to demonstrate digoxin-triggered LNCaP cell apoptosis. In Fig. 6C, treatment of digoxin resulted in LNCaP cell apoptosis (Fig. 6C, green signals in right panels). In contrast, digoxin-triggered cell apoptosis was significantly decreased by co-treatment with BL-I. The quantified data of the TUNEL results are shown in Fig. 6D. Treatment of siRNA-Cdk5 was also shown to reduce digoxin-triggered LNCaP cell apoptosis, although knock-down of Cdk5 expression itself could induce a slight apoptosis (data not shown). As a result, our data show that digoxin triggers Cdk5-related apoptosis in LNCaP cells.

DISCUSSION

Prostate cancer is a worldwide malignant cancer in men. Although early diagnosis contributes to the potential cure of

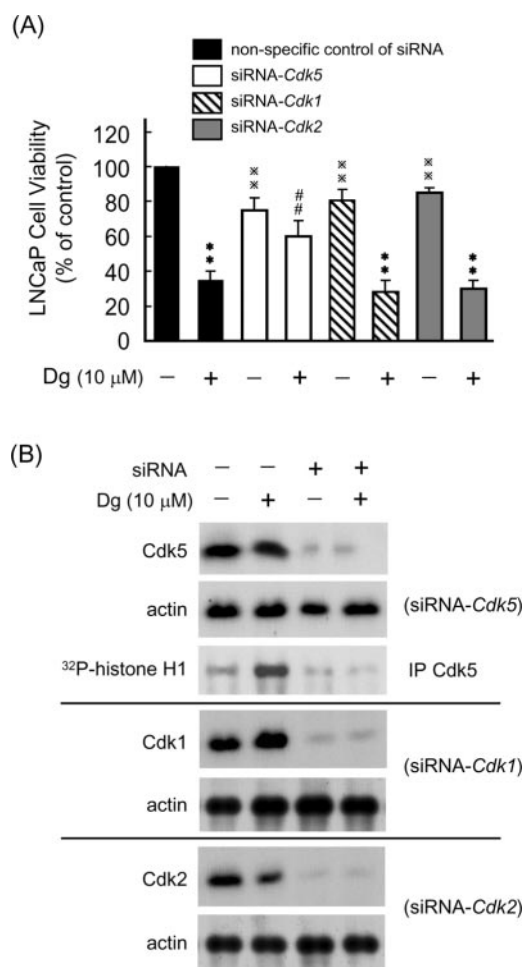


FIG. 5. Digoxin-triggered cell death is Cdk5 protein-dependent. Effects of Cdk5 protein knock-down on digoxin-triggered LNCaP cell death were evaluated after 2-day treatment. A, cell viability was measured by MTT assay as described under "Materials and Methods" ($n = 4$). Digoxin was added in culture medium after transfection of siRNA for 24 h. Black columns represent nonspecific siRNA-treated group; white columns represent siRNA-Cdk5-treated group; hatched columns represent siRNA-Cdk1-treated group; gray columns represent siRNA-Cdk2-treated group. Control value = 100%; **, $p < 0.01$ versus nonspecific siRNA-control group; #, $p < 0.01$ versus nonspecific siRNA-digoxin group. B, cell lysates were subjected to Cdk5 immunoprecipitation followed by anti-Cdk5 immunoblotting and *in vitro* [32 P]ATP kinase assay using histone H1 as the substrate. Cdk1 and Cdk2 protein expressions were also detected by immunoblotting as efficiencies of siRNAs.

localized prostate cancer, metastasis still occurs in many patients. Because the prostate is an androgen-dependent organ, androgen ablation is a priority for treatment. There are many ways of androgen ablation for metastatic prostate cancer, including surgical castration, oral estrogen therapy, luteinizing hormone-releasing hormone analogue depot injection, and other anti-androgen therapies. Besides hormone manipulation, chemotherapy and/or combined therapies were also clinically applied; however, prostate cancer has gradually become androgen-independent and refractory to hormonal therapy (25). Therefore, exploring the novel molecular therapeutic targets by taking the approach of prostate cancer biology is an important issue.

Digoxin is a traditional medicine used for cardiac failure disease. Digitalis, including digoxin, has been considered to be a drug for breast cancer therapy (4, 5). Our lab followed the trail of digitalis and identified its effects upon reproductive endocrinology and prostate cancer cells (6, 26-28). Our previous results suggest that digoxin not only specifically inhibits

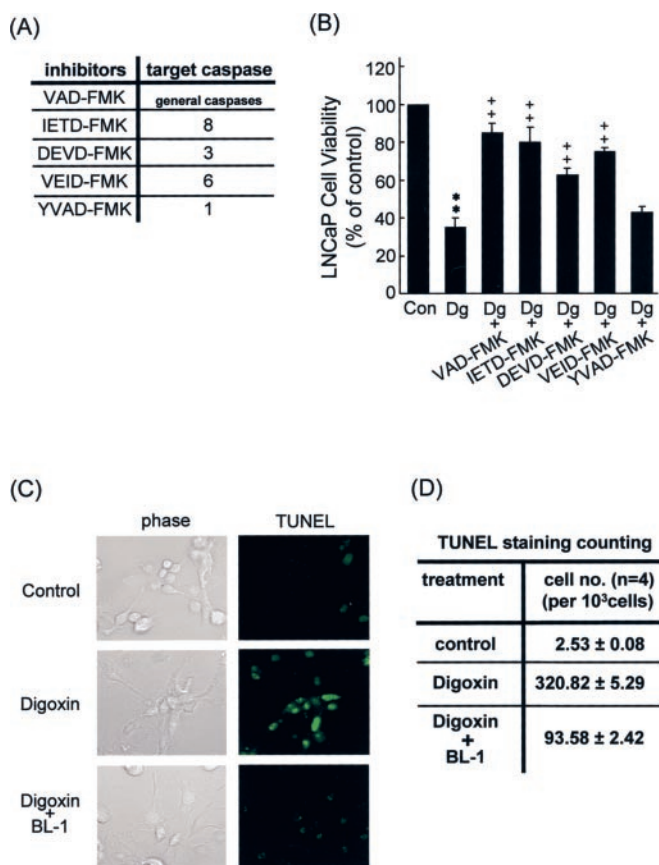


FIG. 6. Digoxin-triggered LNCaP cell death is Cdk5-related apoptosis. A, caspase inhibitor set (1:1000 dilution, Chemicon) was used to treat LNCaP cells with or without digoxin (10 μ M) for 2 days. B, cell viability was measured by MTT assay as described under "Materials and Methods" ($n = 4$). Control value = 100%; **, $p < 0.01$ versus control group; ++, $p < 0.01$ versus digoxin group. C, TUNEL staining (Roche Applied Science) of LNCaP cells was done after treatment with digoxin or digoxin + BL-1 for 2 days. Right panels, green signals show apoptotic cells as compared with the phase image in left panel. Magnification, 80 \times . D, cell number counting per 10³ cells was performed through fluorescence microscopy.

testosterone biosynthesis through the reduction of cAMP production and cytochrome P450_{scc} activity (26), but it also induces cytotoxicity of prostate cancer cells (6). These results imply the dual effects of digoxin upon the reduction of prostate cancer development. In addition, McConkey *et al.* also addressed the involvement of digoxin in prostate cancer cell death (29). Therefore, the detailed mechanisms of digoxin in prostate cancer cell death become interesting subjects to be investigated further for potential cancer therapy.

Cdk5, a non-cell-cycle-related cyclin-dependent kinase, is believed to function in the central nervous system along with its regulator, p35 (30). Several models for neuronal death indicate the involvement of Cdk5 hyperactivation, such as Alzheimer's disease and amyotrophic lateral sclerosis (31, 32). A typical hypothesis is that various signaling pathways trigger the rise of intracellular Ca²⁺ and result in general oxidative stress (18). One of the downstream effectors is p35 cleavage, which leads to the accumulation of p25 and Cdk5 activation (10). Therefore, activation of Cdk5 by digoxin-induced p25 formation becomes a reasonable interpretation. First, we identified intracellular Ca²⁺ to be important for toxic effects of digoxin by chelation of Ca²⁺ in culture medium. Eliminating the influence of Ca²⁺ did effectively prevent prostate cancer cell death, which was usually caused by digoxin in two different prostate cancer cell lines (Figs. 1A and 2A).

It has been demonstrated that p25 is a pathological signal in several neurodegenerative diseases (10, 33). Generally, oxidative stress could increase intracellular Ca²⁺ concentration and stimulate calpain activity (10). Tsai *et al.* (32, 33) at Harvard Medical School reported that activated calpain cleaves neuronal p35 into p25, which is then able to hyperactivate Cdk5 and subsequently leads to hyperphosphorylation of cytoskeleton-associated protein tau, which is the cause of neurofibrillary tangle and also a hallmark of Alzheimer's disease. Although hyperphosphorylation of tau is believed to be a downstream cause in neurodegenerative diseases, much evidence indicates that Cdk5 could result in apoptosis directly (23, 24). In addition, androgen withdrawal-induced regression of the prostate gland in male mice was correlated to Cdk5-related apoptosis (14). These reports imply that Cdk5-related apoptosis could happen in any tissues where Cdk5/p35 and p25 exist. Here, we not only first identified that Cdk5, p35, and p25 were all simultaneously expressed in prostate cancer cells, but also found the production of p25 was enhanced by treatment of digoxin and paralleled Cdk5 activation (Figs. 1B and 2B).

To investigate further the relation of p25 formation and cell death, p35 cleavage inhibitor was used. The result indicated that p25 formation was the primary cause of digoxin-triggered cell death. This finding implied that p25-dependent activation of Cdk5 might play a vital role in digoxin-triggered cell death (Fig. 3). Therefore, we tested the role of Cdk5 activity in digoxin-triggered LNCaP cell death by the administration of two different Cdk5 inhibitors (Fig. 4). In a recent report (22), RV was used to inhibit Cdk5 kinase activity in non-neuronal cells (pancreatic β cells). This suggests the feasibility of using these kinase inhibitors in Cdk5-expressed cells. The administration of Cdk5 inhibitors and p35 cleavage inhibitor both reduced digoxin-induced Cdk5 activation and significantly prevented digoxin-triggered LNCaP cell death. These results directly or indirectly suggest digoxin-induced Cdk5 activation is involved in digoxin-triggered cell death. In addition, siRNA-Cdk5 was manipulated to show that reduction of Cdk5 protein expression could significantly prevent cell viability caused by digoxin as compared with the results of the treatment of siRNA-Cdk1 or siRNA-Cdk2 (Fig. 5A). These data also offset the results of Cdk5 inhibitors, which cross-react with Cdk1 and Cdk2 (see Fig. 4). Moreover, the reduction in Cdk5 protein expression alone affected cell viability, as compared with the nonspecific siRNA control (Fig. 5A). This result implies a novel physiological role for Cdk5 in LNCaP cell proliferation. It is also an indication that Cdk5/p35 functions in prostate cancer cells and regulates prostate cancer cell death under digoxin treatment.

Based upon the involvement of Cdk5 in digoxin-triggered cell death, the next question we encountered was whether this Cdk5-related cell death is apoptosis. To decide this issue, a set of caspase inhibitors (Chemicon) was used to screen the preventative effects of these inhibitors on digoxin-triggered cell death. Our results showed different prevention percentages of digoxin-triggered cell death contributed by different caspase inhibitors (Fig. 6B). Inhibitors of general caspase (VAD-fmk), caspase-8, caspase-6, and caspase-3, were found to have a stronger preventative effect compared with that of the caspase-1 inhibitor. This finding indicates that digoxin causes LNCaP cell death through multi-caspase-dependent apoptosis, as compared with a previous report (29). We further identified digoxin-triggered LNCaP cell death by TUNEL staining; the apoptosis was also proved to correlate to Cdk5 activity (Fig. 6, C and D). Although the results were all performed in LNCaP cells, other prostate cancer cells, such as PC-3 or DU-145, were also tested. Similar results were observed (data not shown to avoid reiteration). In other cell types, such as MRC-5 (human embry-

onal lung fibroblast) or Hep-G2 (human hepatoblastoma), without an abundant expression of p35, the minor effects of cytotoxicity caused by digoxin were observed (data not shown). Taken together, we suggest that Cdk5/p35,p25 were specific molecular targets of digoxin treatment in prostate cancer cells. Because the prostate gland is an androgen-dependent organ, digoxin *in vivo* is found able to not only inhibit testosterone production (26) but also directly induce prostate cancer cell apoptosis. Thus, digoxin would become a novel and potential therapeutic strategy.

In conclusion, digoxin triggers prostate cancer cell apoptosis, at least through the Cdk5/p35,p25-dependent pathway, which is responsive to an increase of intracellular Ca^{2+} . We have also demonstrated the expressions of Cdk5/p35,p25 in prostate cancer cells as well as the presence of Cdk5/p25-related apoptosis. Our finding is a distinctive example in which Cdk5-related regulation does not happen in the central nervous system. The physiological functions of Cdk5/p35 in the prostate gland and cancer await investigation. However, our results reveal the novel mechanisms of digoxin-induced prostate cancer cell apoptosis and shed light on the possibility of cancer therapy.

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